AD_____

Award Number: W81XWH-05-1-0594

TITLE: Development of the C-Terminal Inhibitors of Heat Shock Protein 90 in the Treatment of Prostate Cancer

PRINCIPAL INVESTIGATOR: Jeffrey Holzbeierlein

CONTRACTING ORGANIZATION: The University of Kansas Medical Center Research Institute
Kansas City, KS 66160-7702

REPORT DATE: October 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 Oct 2008	Final	15 Sep 2005 – 14 Sep 2008

4. TITLE AND SUBTITLE

Development of the C-Terminal Inhibitors of Heat Shock Protein 90 in the Treatment of Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0594

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Jeffrey Holzbeierlein

E-Mail: jholzbeierlein@kumc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Kansas Medical Center Research Institute
Kansas City, KS 66160-7702

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Purpose: To develop and evaluate novel compounds which inhibit the C-terminal portion of Hsp90 in prostate cancer models. Scope: To develop a library of novobiocin analogues to be tested in vitro initially and then in a mouse model. Major findings: we have tested over 220 analogues of the original parent compound (A-1) all of which have been tested in at least two commonly used prostate cancer cell lines LNCaP and PC3. Screening has been evaluated by both Western Blot of client proteins which include the androgen receptor, Akt, Hsp90, and HER2, and MTT assay. Furthermore, with our most effective compounds we have examined the mechanism of action and have examined cell cycle data. We have evaluated one compound in vivo and are currently evaluating our newest most promising compound in vivo in an orthotopic mouse model.

15. SUBJECT TERMS
Prostate cancer, Heat Shock Protein 90

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	26	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Introduction……..5

BODY……5

Key Research Accomplishments…….. 8

Reportable Outcomes…… 8

Conclusion…… 9

References……9

Appendices…… 10

Introduction: A great deal of interest in the development of inhibitors of Heat Shock Protein 90 as anticancer agents exists. As the client proteins of Hsp90 include the androgen receptor as well as many other proteins believed to be critical for prostate cancer development and progression, these agents have potentially significant use against prostate cancer. Current inhibitors of Hsp90 (the N-terminal inhibitors) have been limited in their clinical usefulness due to toxicity, poor solubility, and difficulty in synthesis. Multiple Phase II trials are ongoing to evaluate these N-terminal inhibitors but results are limited. Our research focuses on a group of novel compounds which inhibit the C-terminal portion of Hsp90 and may be an effective and less toxic treatment for prostate cancer.

Body:

Specific Aim #1 To test the novel compound KU-1 in several prostate cancer cell lines to assess the effect of the drug on proteins known to be involved in prostate oncogenesis. In addition, apoptotic assays will be performed at different concentrations to assess cell death.

KU-1, a novel analogue of novobiocin, which had been previously tested in SkBr3 cells was applied to three prostate cancer cell lines, LNCaP, PC3, and LAPC-4 cells. Each cell line was tested due to its unique properties, LNCaP most widely used and androgen dependent, PC3 a commonly used androgen insensitive cell line, and LAPC-4 an androgen sensitive cell line with a wild type androgen receptor. Western blots for the client proteins, androgen receptor, Akt, HIF1α, and HER2 were obtained. (Figure1) Actin was used as a control as it is generally not believed to be a client protein of Hsp90.

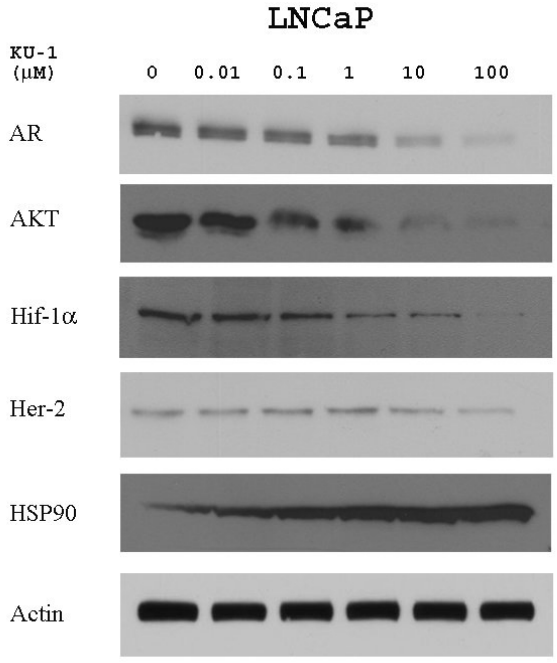
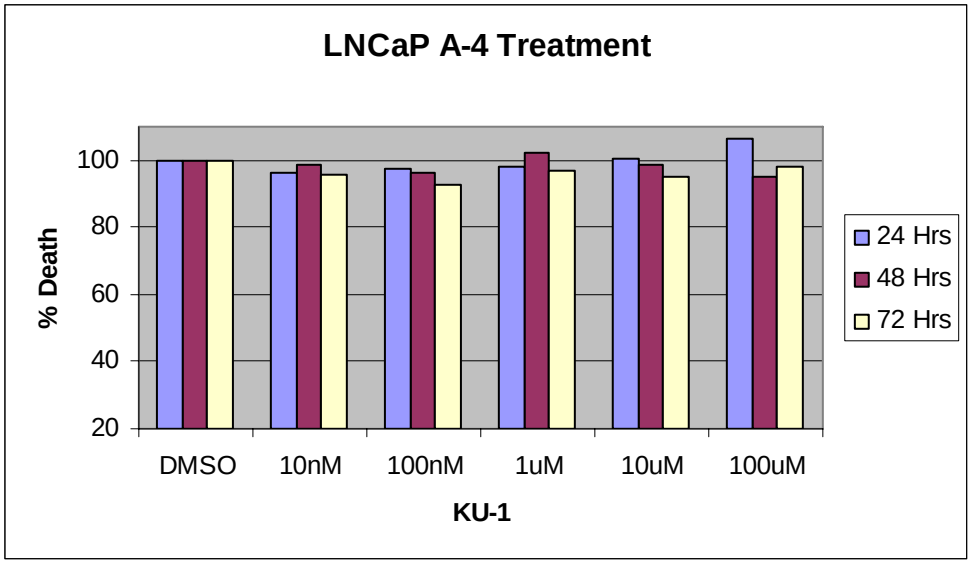


Figure 1

These results demonstrate an effect of KU-1 at approximately the 10uM range. This is significantly more potent than its parent analogue Novobiocin. Similar results were then obtained when KU-1 was applied to LAPC-4 and PC-3 (graph not shown). An MTT assay was then conducted on LNCaP cell using KU-1 (also called A-4) at various concentrations and for different time periods. Results are seen in Figure 2.



These results show that KU-1 does not appear toxic to LNCaP cells even at concentrations of 100uM. Interestingly, this has applications for other disease states such as Alzheimers but toxicity through inhibition is something desirable for cancer studies. Therefore, we believe that this compound has the ability to inhibit Hsp90 at relatively lower concentrations but in and of itself is not toxic to cancer cells. This research was completed in the stated time period of 4 months.

Because of the time, work and expense of the synthesis of this compound and its analogues it was decided that more potent analogues of KU-1 would be developed prior to their testing in an in vivo model. This is the aim of Task 3 and will be described in detail.

The compound which was initially selected based on its MTT assay results and Western blot results was KU-36 (F-4 in manuscript). A manuscript which has been previously submitted to Cancer Research but was not accepted, and has now been revised and will be sent to the Journal of Urology is attached which details the results of the in vitro studies with KU-36. Appendix 1.

<u>Specific Aim #2:</u> To test the effectiveness of KU-1 in an LNCaP nude mouse xenograft model and determine the toxicity of the compound.

As mentioned previously due to the expense and time commitment required to scale up these compounds to sufficient quantities to be used in an in vivo model, we chose not to test KU-1 in vivo as we had identified KU-36 as having increased potency in vitro. Furthermore, we chose to evaluate this compound in an orthotopic mouse model using PC-3 cells which more closely

resembles the human condition of hormone refractory prostate cancer, a condition where a compound of this nature would have its greatest use.

Animals were given KU-36 in the following dose concentrations with the following dosing schedule. An orthotopic mouse model using PC-3 cells in nude mice was established. Mice were treated with KU-36 at doses of 1, 5, 25, and 75 mg/kg. The animals were given 2 cycles consisting of daily doses for five days for a total of ten doses. Dosing was started on day 7 and continued for five days, two days off then five more days and sacrificed the animals on day 21.

Tumor take rate for the orthotopic model was 100% (all animals manifested tumors in the prostate). A control group of untreated animals (with tumors) was also included and used for tumor growth comparison. Animals were monitored for toxicity and blood obtained at 2 time points for pharmacokinetic data (results pending at this point). No appreciable toxicity was observed in the animals at any time point or dosage. Preliminary results show no appreciable tumor shrinkage with KU-36 in the current dosage and treatment schedule. Tumors were harvested and are currently undergoing evaluation for the effect of the treatments on client proteins in the tumors. In addition, since we have identified a new compound with greater potency through screening in Aim #3 we are scaling up this compound for another in vivo experiment.

<u>Specific Aim #3:</u> To develop analogues of KU-1 with even greater potency.
A library of novobiocin analogues has been prepared with different modifications made in a sequential fashion based on results of Western data and MTT data which tend to parallel each other well except for KU-1 which showed little toxicity. A list of IC-50's of the 10 most potent compounds trested to data is presented in Table 1.

Rank Order of Top Ten KU Compounds Based on Percent Efficacy and EC50 data

TABLE ONE: [1] = first generation compound, [2] = second generation compound

Rank	Compound	LnCaP Ave. EC50 (M)	St. Dev.	LnCaP % Efficacy	St. Dev.	PC-3 Ave. EC50 (M)	St. Dev.	PC-3 % Efficacy	St. Dev.
1	174 [2]	1.21E-07	0.02	96.25	1.42	1.83E-07	1.0E-07	82.46	16.20
2	146 [1]	1.47E-06	3.97E-07	93.61	1.93	2.28E-06	1.3E-06	76.56	8.26
3	127 [1]	2.46E-06	1.11E-06	79.89	1.69	4.77E-06	4.4E-06	82.20	5.94
4	123 [1]	2.27E-06	2.08E-07	74.91	3.33	4.77E-06	3.3E-06	68.93	7.53
5	131 [1]	1.70E-06	7.23E-07	74.28	11.34	4.44E-06	1.2E-06	57.11	11.33
6	139 [1]	1.22E-06	1.81E-07	61.66	7.66	9.32E-06	n/a	57.28	n/a
7	124 [1]	4.54E-06	9.82E-07	52.90	9.19	3.89E-06	6.9E-08	56.85	22.23
8	137 [1]	5.21E-06	3.81E-06	60.48	17.81	4.41E-06	1.0E-05	43.35	1.39
9	135 [1]	7.01E-07	2.21E-07	80.28	4.12	4.14E-07	1.4E-07	64.29	5.18
10	150 [1]	1.87E-06	6.69E-07	92.74	5.33	4.14E-06	1.79E-06	76.68	10.12
1st generation compound	36 [1]	1.42E-05	1.12E-06	34.39	8.59	2.19E-05	4.57E-06	16.31	3.17
Parent molecule	Novobiocin	2.01E-04	31.40	-19.17	10.18	2.18E-04	4.0E-05	-0.31	3.81
N-terminal Hsp90-I	17-AAG	5.72E-08	4.11E-10	74.02	2.88	7.52E-08	6.3E-09	80.77	5.01

We have currently tested over 220 analogues of our parent compound, and continue to test analogues.

Key Research Accomplishments: Confirmation of KU-1 as an inhibitor of key client proteins involved in prostate cancer oncogenesis

Preparation of a library of novobiocin analogues with greater potency than KU-1 and will cellular toxicity as measured by

Western blot and MTT assay.

Demonstration of an effect of the C-terminal inhibitors activity in an androgen independent cell line

Confirmation of in vivo non toxic nature of these compounds

Future Plans: We have continued to identify newer analogues with greater potency. We will also plan to test these agents in vivo and explore different dosages and dosing schedules. Furthermore, we will also evaluate different tumor models i.e. using an LNCap orthotopic cell model.

Reportable Outcomes: Yu XM, Shen G, Neckers L, Blake H, Holzbeierlein J, Cronk B, Blagg BS Hsp90 inhibitors identified from a library of Novobiocin analogues. J Am Chem Soc. 2005 Sep 21;127(37):12778-9.

Donnelly AC, Mays JR, Burlison JA, Nelson JT, Vielhauer G, Holzbeierlein J, Blagg, BS The Design, Synthesis, and Evaluation of Coumarin ring Derivative of the Novobiocin Scaffold that Exhibit Antiproliferative Activity. J. Org Chem epub ahead of print 10/22/08

Goetzl M, Blagg BS, Cronk B, Neckers L, Holzbeierlein JM. Modulation of Heat Shock Protein 90 (Hsp90) Client Protein Expression in Prostate Cancer Cells by a Novel Novobiocin Analog. J Urol 2006 175,4:140

Presentations: 2005 Society for Urologic Oncology Winter Meeting, NIH Bethesda, MD"Modulation of Heat Shock Protein 90 (Hsp90) Client Protein Expression in Prostate Cancer Cells by a Novel Novobiocin Analog."-Poster presentation

2006 American Urological Association Meeting, Orlando FL "Modulation of Heat Shock Protein 90 (Hsp90) Client Protein Expression in Prostate Cancer Cells by a Novel Novobiocin Analog.". Poster Presentation

2007 Society for Urologic Oncology Winter Meeting NIH Bethesda MD "Development of C-Terminal Inhibitors of Heat Shock Protein 90 in the Treatment of Prostate Cancer." Poster Presentation

Conclusions: To date we have confirmed that a novel novobiocin analogue which inhibits the C-terminal portion of Hsp90 results in down-regulation of client proteins believed to be important in prostate cancer oncogenesis. Specifically, Akt, androgen receptor, HER2 and HIF1α proteins are down-regulated in LNCaP and LAPC-4 cells. In addition, this is seen in an androgen independent cell line PC-3 which may have particular significance in patients with androgen independent prostate cancer.

A library of analogues of KU-1 has been prepared and has likewise been tested in several prostate cancer cell lines. Many of these analogues show significantly increased potency in inhibiting Hsp90 client proteins, particularly KU-174 and KU-146. Furthermore, these compounds exhibit an IC50 in the low micromolar to possibly nanomolar range.

Although our initial in vivo data did not demonstrate significant tumoricidal effects, there was no significant toxicity observed with the compound. In addition, we are still assessing the effect of the compound on the client proteins in the tumors of the animals treated with compound. As we have identified compounds with greater potency we are currently scaling up our most potent compound to date 174, to test in our in vivo model.

"So What" These agents have great potential for the treatment of prostate cancer particularly in the androgen independent state. Alone or in combination with other chemotherapy agents, these compounds represent a *novel* and exciting approach to prostate cancer. In addition, our early experience with these agents suggests that they may have a very good tolerability profile in terms of minimal toxicity.

List of Personnel

Brian Blagg, PhD
Benjamin Cronk
Chris Avila

Appendix 1

Characterization of a novel novobiocin analogue as a putative C-terminal inhibitor of heat shock protein 90 in prostate cancer cells.

Shawna B. Comer[1#], George A. Vielhauer[2#], Craig A. Manthe[1#], Vamsee K. Chaguturu[2#], Christopher Avila[1], Benjamin B. Cronk[1], Xiao Ming Yu[8], Joseph A. Burlison[8], Katherine F. Roby[3], John D. Robertson[4] , Mark S. Cohen[5], Scott J. Weir[2], Michael B. Hughes[2], Melinda A. Broward[2], Roger A. Rajewski[6], Len M. Neckers[7], Brian S. J. Blagg[8], and Jeffrey M. Holzbeierlein[1#]*
(The University of Kansas Hsp90 C-terminal Inhibitor Research Consortium)

[1]Department of Urology, University of Kansas Medical Center, Kansas City, KS
[2]University of Kansas Cancer Center, University of Kansas Medical Center
[3]Department of Anatomy and Cell Biology, University of Kansas Medical Center
[4]Department of Pharmacology, Toxicology, and Therapeutics, University of Kansas Medical Center
[5]Department of Surgery, University of Kansas Medical Center
[6]Department of Higuchi Biosciences Center, University of Kansas, Lawrence, KS
[7]Cell and Cancer Biology Branch, National Cancer Institute, NIH, Rockville, MD
[8] Department of Medicinal Chemistry, University of Kansas

[#]contributed equally

*Address Correspondence to:
Jeffrey M. Holzbeierlein, MD
Associate Professor of Urology
University of Kansas Medical Center
3901 Rainbow Blvd, Mail Stop 3016
Kansas City, KS 66160
Running title: Design of Hsp90 inhibitors for prostate cancer.
Keywords: Hsp90 inhibitors, prostate cancer, novobiocin

ABSTRACT

Purpose: Hsp90 is important in the folding, maturation and stabilization of client proteins critical to cancer cell survival. Thus, Hsp90 represents a viable drug target for which the design of inhibitors has therapeutic potential in cancer. We have previously reported the development of novobiocin analogues designed to inhibit the C-terminal portion of Hsp90. We hypothesized that by targeting the Hsp90 C-terminal domain, the novobiocin analogue, F-4, will demonstrate a distinct mode of action and improved cytotoxicity in prostate cancer cells compared to the N-terminal inhibitor, 17-AAG.

Materials and Methods: LNCaP and PC-3 cells were treated with 17-AAG or F-4 in anti-proliferative and cytotoxicity assays. Western blots and prostate specific antigen (PSA) ELISA was examined after F-4 treatment to determine client protein degradation, the induction of Hsp90 and assess the functional status of the androgen receptor (AR). Lastly, the induction of apoptosis and disruption of cell cycle was examined following F-4 and 17-AAG treatment.

Results: F-4 demonstrated improved potency and efficacy compared to novobiocin in anti-proliferative assays and decreased expression of client proteins. Interestingly, PSA secrection was reduced in a dose-dependent manner that paralleled a decrease in androgen receptor expression. Suprisingly, F-4 had superior efficacy to 17-AAG at inducing cytotoxicity, apoptosis and cell cycle arrest.

Conclusion: F-4 represents a novobiocin analog with enhanced activity compared to the parent molecule and 17-AAG in prostate cancer cells. These data reveal differences in the mode of action when comparing N-terminal and C-terminal Hsp90 inhibitors in prostate cancer cells with the latter being more cytotoxic.

INTRODUCTION

Prostate cancer remains a significant health problem in the United States, responsible for 30,000 deaths annually, and is the second leading cancer killer of men[1]. Patients with metastatic, locally recurrent, and androgen-independent prostate cancer are particularly problematic, and though recent trials demonstrate limited activity for docetaxel in these cases, the effectiveness of chemotherapy remains limited[2, 3]. Prostate cancer remains a

heterogeneous disease with multiple contributing pathways, and future treatment strategies must focus on the design of single agents with the ability to target multiple pathways.

Heat shock protein 90 (Hsp90) is a molecular chaperone responsible for folding nascent polypeptides (client proteins) into their biologically active conformations. In cancer, Hsp90 facilitates the conformational maturation of dormant oncogenic proteins important to tumorigenesis and cancer cell survival [4, 5]. Additionally, tumor cells exhibit higher Hsp90 activity and increased accumulation of Hsp90 inhibitors compared to normal cells, which may allow for the design of tumor-selective inhibitors[6, 7]. In contrast to traditional cancer therapuetics directed against a single molecular target, disruption of the Hsp90 machinery may simultaneously inhibit multiple therapeutic targets and pathways critical to tumor survival. Thus, Hsp90 inhibitors represent an exciting new strategy in the development of prostate chemotherapies.

Traditionally, Hsp90 inhibitors have targeted the N-terminal ATP-binding site; more recently, a second ATP-binding site was identified in the carboxyl terminus of Hsp90[8]. Inhibition of the Hsp90 C-terminus decreases chaperone dimerization, diminishes ATPase activity and impairs formation of the Hsp90 complex with client proteins, resulting in improperly folded proteins that are targeted for the ubiquitin-proteasomal degradation pathway[9]. The ansamycin antibiotic novobiocin has been demonstrated to bind to the C-terminal site of the Hsp90 molecular chaperone[10, 11]. Clinically, novobiocin has been used for its antimicrobial activity with acceptable toxicity and bioavailability. Unfortunately, it exhibits low Hsp90 affinity with an IC_{50} of ~400 μM and would require high concentrations for maximal effects[10, 11]. Thus, we hypothesize novobiocin analogues with improved affinity for Hsp90 may represent effective therapy for prostate cancer. We have recently reported the synthesis and screening of novobiocin analogues designed to bind to the Hsp90 C-terminal domain [10, 12]. Herein, we report the characterization of a previously unreported analogue, F-4, in prostate cancer cell lines.

MATERIALS AND METHODS

Materials: Novobiocin analogues were synthesized as previously described[13]. F-4 was dissolved in DMSO and stored at -20°C until use. Commercial antibodies were obtained for the androgen receptor (AR) (Cell Signaling

Technologies, Danvers, MA), AKT, Hsp90, HER-2, HIF-1α and Actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA), Hsp70 and GAPDH (Cell Signaling Technologies, Danvers, MA).

Cell culture: LNCaP and PC-3 prostate cancer cell lines were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 and Ham's F-12 media, respectively, with 10% FBS and penicillin/ streptomycin (100 IU/ml/ 100ug/ml) and maintained at 37°C with 5% CO_2.

Anti-proliferative assay: This assay was performed as previously described[14]. Cells were incubated for 48 hours incubation then treated for the specified timepoints. The CellTiter 96 AQueous One Solution Cell Proliferation Reagent (Promega) was used according to manufacturer protocol. Briefly, cell proliferation was measured by metabolism of a tetrazolium (MTS) dye to a formazan by-product, which corresponds to viable cell number as determined by metabolic ability. Plates were analyzed on the VICTOR^{3}V Multilabel Reader (PerkinElmer) at 490 nm. Data was analyzed from 2-4 independent experiments performed in duplicate then normalized to absorbance of media only, and non-linear regression and sigmoidal dose-response curve (GraphPad Prism) were used to calculate IC_{50} and IC_{90} values.

Western Blot analysis: Western blot was performed as previously described[15]. Briefly, cells were treated with F-4 or vehicle (DMSO) for indicated times. Lysates were prepared, and equal amounts of protein electrophoresed under reducing conditions on 4-12% Tris-Glycine gels (Invitrogen, Carlsbad, CA), transferred to a PVDF membrane (Millipore, Bedford, MA) then blocked in TBS-T containing 5% milk and probed with primary antibodies. Membranes were incubated with a horseradish peroxidase-conjugated secondary antibody, developed with chemiluminescence substrate (Pierce Biotechnology, Rockford, IL), and visualized with the UVP AutoChemi system (UVP, LLC, Upland, CA)**.** All Western blots were probed for loading controls Actin or GAPDH. The data are representative from at least three independent experiments (n=3).

PSA ELISA assay: *In vitro* measurement of prostate specific antigen (PSA) secretion from LNCaP cells was assessed using the BioQuant PSA ELISA kit (BioQuant, Nashville, TN) according to manufacturer's instructions. Cells were cultured in medium containing 2% charcoal dextran-stripped serum for 3 days to reduce hormone to basal

levels, then were treated with F-4 for 24 hours. Cells were either incubated with F-4 alone or in the presence of 100 nM testosterone for an additional 24 hours. Following incubation, samples of LNCaP-conditioned media were analyzed for PSA. Data was analyzed as described under **Anti-Proliferative Assay**. Data points represent the mean ± SEM of duplicate wells from independent experiments (n=4).

Annexin V apoptosis experiments: Annexin V-FITC and propidium iodide (PI) (Anaspec, San Jose, CA) were prepared according to the manufacturer's instructions. Cells were treated then floating and adherent cells were collected and the resulting cell suspension was washed in Annexin Binding Buffer (ABB) (150 µM NaCl, 5 µM KCl, 1 µM $MgCl_2 \cdot 6H_20$, 1.8 µM $CaCl_2 \cdot 2H_20$, 10 mM HEPES, 2% FBS). Half of the cell suspension was used for **Cell Cycle Analysis** (see below). The remaining cell suspension was stained with Annexin V-FITC (BD Pharmingen, San Jose, CA) and Propidium Iodide (Sigma Aldrich, St. Louis, MO) then washed and fixed in paraformaldehyde. Samples were analyzed using the BD LSRII System (BD Biosciences, San Jose, CA) and data was analyzed using a t-test with GraphPad Prism 5.0. The data displayed represent the mean ± SEM for three independent experiments (n=3).

Cell cycle analysis: Cells were centrifuged and resuspended in 0.9% NaCl followed by drop-wise addition of 90% EtOH to fix cells. Samples were centrifuged then resuspended in PI followed by incubation with RNase A. All samples were analyzed as described under Annexin V methods. *Columns* depict the mean $\pm$ SEM from three independent experiments (n=3).

Trypan Blue cytotoxicity experiments: Cells were treated for the indicated time then floating and adherent cells were collected. Each sample was mixed with equal parts Trypan Blue and analyzed on the VI-CELL Series Cell Viability Analyzer (Beckman Coulter, Fullerton, CA). Percentage of viable cells was calculated by determining the ratio of Trypan Blue-positive to negative cells. Data represents the average of two independent experiments (n=2).

RESULTS

Anti-proliferative effects of F-4

The anti-proliferative effects of F-4, 17-AAG, and novobiocin were examined over a 72 hours time course in LNCaP and PC-3 cells (*Figure 1*). At all timepoints, F-4 dose-response curves were nearly superimposable with marginal improvement over time indicating maximal efficacy (IC_{90}) and potency (IC_{50}) is essentially achieved at 24 hours (*Table 1*). Conversely, 17-AAG requires at least 48-72 hours treatment to achieve comparable efficacy and potency in both cell lines. F-4 was 14-24 fold and seven to nine fold more potent (IC_{50}) compared to the parent compound novobiocin in the LNCaP and PC-3 cell lines, respectively, at the different timepoints. These findings suggest that the novobiocin analogue, F-4, requires less time to exert its mode of action compared to 17-AAG which requires 48-72 hours of continuous exposure to elicit its maximal response.

F-4 mediated degradation of client proteins.

Client protein expression and subsequent degradation was examined in LNCaP cells treated for 24 hours with F-4. Reduced expression of AR, HER-2 and AKT proteins was observed in a dose-dependent manner (*Figure 2A).* HIF-1α and Hsp90 were largely unaffected and Actin was marginally affected at the highest concentration.

PC-3 cells, demonstrated decreased AKT and HER-2 protein expression (*Figure 2B)* while Hsp90 expression was moderately increased following 72 hours F-4 treament. HIF-1α appeared to be more sensitive to F-4 as client protein degradation was observed following 24 hours of treatment. These data demonstrate a hallmark of Hsp90 inhibition, the degradation of client proteins important in prostate cancer, and suggests F-4 disrupts the Hsp90 folding machinery.

F-4 abolishes androgen mediated PSA secretion

The expression and secretion of PSA is regulated by androgens via binding to their cognate androgen receptor (AR). Since, F-4 decreases AR expression between one and ten micromolar (*Figure 2A)* we examined whether there was a corresponding effect on the secretion of PSA in the androgen dependent cell line using charcoal stripped media. Treatment of cells with testosterone alone demonstrated a robust increase in PSA that was abrogated with pretreatment of F-4 at similar concentrations required to induce AR degradation *(Figure 3)*. These data demonstrate

that degradation of AR can be monitored by PSA level providing an important biomarker for assessing the effectiveness of C-terminal Hsp90 inhibitors in androgen dependent prostate cancer.

Induction of apoptosis by F-4 in prostate cancer cells.

Induction of apoptosis was determined by Annexin V binding to externalized phosphatidylserine on the outer leaflet of the plasma membrane and cell death measured by propidium idodide staining. Treatment of prostate cancer cells with F-4 resulted in a dramatic dose-dependent induction of Annexin V staining compared to control (*Figure 4A & 4C*). Interestingly, the LNCaP cell line *(panael 5A)* appeared to undergo early-stage (quadrant IV) and late-stage (quadrant II) apoptosis increasing with dose while the less sensitive PC-3 cell line *(panael 5B)* was only observed to undergo early apoptosis (quadrant II) with increasing dose. Statistical analysis of these data revealed a significant increase in Annexin V staining at the 50 ($p<0.05$) and 100 μM ($p<0.01$) F-4 doses as compared to control (*Figure 4B & 4D, respectively*). Additionally, 17-AAG at concentrations of 12.5 and 20 fold higher than the IC_{50} from the anti-proliferative assays in PC-3 and LNCaP cells, respectively, failed to match the induction of F-4 mediated early and late-stage apoptosis at concentrations 1.5-4 fold higher than the IC_{50} for F-4. These findings demonstrate F-4 to be more effective at inducing apoptosis in prostate cancer cells compared to 17-AAG.

F-4 Disruption of cell cycle in prostate cancer cells.

Cell cycle arrest was assessed in both prostate cancer cell lines following F-4 treatment. In LNCaP cells we revealed a marked increase in the sub-G0 percentage of cells *(Figure 5A)* with a decrease in the percentage of cells in the G0/G1 and G2/M phase *(Figure 5B).* The sub-G0 peak represents cells undergoing apoptosis, characterized by DNA fragmentation and reduced cellular DNA. Conversely, cell cycle analysis of PC-3 cells following exposure to F-4 resulted in a G2/M arrest (*Figure 5C*) with a corresponding decrease in the percentage of cells in the G0/G1 phase of the cell cycle (*Figure 5D*). Interestingly, treatment of both cell lines with 17-AAG resulted in an initial G2/M arrest with no subsequent induction of sub-G0 phase *(Figure 5B & 5D).* These data reveal that F-4 has distinct effects on cell cycle in the two prostate cancer cell lines while 17-AAG behaves in an identical manner.

Cytotoxicity of F-4 in prostate cancer cells.

Since the anti-proliferation assays measure only non-proliferating metabolically inactive cells and not cytotoxicity, cells were examined using trypan blue experiments to discern whether F-4 and 17-AAG were cytotoxic. LNCaP *(Figure 6, top panels)* and PC-3 *(Fiugre 4, bottom panels)* cells were treated for 24 *(panels A & D)*, 48 *(panels B & E)*, or 72 *(panels D & F)* hours, and viability was measured using the Trypan Blue exclusion assay. A dose dependent decrease in cell viability was observed at concentrations of 25-50 μM and 75-100 μM in LNCaP and PC-3 cells, respectively, in comparison to 17-AAG. Thus, at concentrations 625 and 1000 fold higher than the anti-proliferative IC_{50} for 17-AAG *(Table 1)* the compound demonstrates little cytotoxicity whereas, at concentrations 1.5-4 fold higher than the anti-proliferative IC_{50} for F-4 we observe pronounced cytotixcity suggesting analogues of novobiocin are superior at inducing cell death in prostate cancer cells.

DISCUSSION

Hsp90 is a molecular chaperone required for the folding of nascent and denatured proteins. In the absence or inactivity of Hsp90, these client proteins remain inactive, leading to ubiquitination and proteasomal degradation. Though several N-terminal Hsp90 inhibitors have been shown to be effective in causing client protein degradation, many of these agents have been hampered in clinical trials by high toxicity and poor solubility[16]. The C-terminal ATP binding domain of Hsp90 is important for dimerization and can be inhibited by novobiocin. We hypothesized that novobiocin analogues targeting Hsp90 would demonstrate a distinct mode of action compared to N-terminal inhibitors.

Herein, we report the pharmacological profile of the novobiocin analog, F-4, in comparison to 17-AAG. In anti-proliferation assays, F-4 demonstrates superior efficacy (IC_{90}) at 24 hours exposure compared to 17-AAG which requires 48-72 hours to achieve maximal response. From these data, concentrations were selected around the IC_{90} for F-4 (25-100 μM) and 17-AAG (one micromolar) to test in other functional assays. A hallmark of Hsp90 inhibition is the degradation of client proteins, we therefore investigated the expression of client proteins which have been established to be important in prostate cancer. cancer cell 2003 neckers p213, clin can res 2004, p6572, and Best Pract Res Clin Endocrinol Metab. 2008 Apr;22(2):357-72).

F-4 treatment of prostate cancer cells demonstrated some client proteins (HER-2 and AKT) were affected across cell lines but another client, HIF-1α, was only affected in the PC-3 cell line suggesting not all proteins will be equally degraded following Hsp90 inhibition across prostate cancers. Interestingly, the induction of Hsp90, a hallmark of N-terminal Hsp90 inhibition, is not observed in the LNCaP cell line and mildly observed in the PC-3 cell line. To date, it is unclear how Hsp90 induction will affect the clinical outcomes of N-terminal Hsp90 inhibitors. However, if it is proven to be undesirable, novobiocin analogues targeting the C-ternimal end of Hsp90 may offer distinct advantages in that they limit the induction of Hsp90. Correspondingly, PSA levels decline at concentrations (10μM) less than what is required for cytotoxicity (25-100 μM) indicating that the reduction is a result of AR degradation and not cell death. Thus, PSA levels serve as a valuable biomarker that can be used in androgen dependent cancers to assess the activity of Hsp90 inhibitors *in vivo*.

In studies examining cell cycle, induction of apoptosis and cytotoxicity of F-4 and 17-AAG, we note several key observations. First, F-4 appeared to have distinct effects on the cell cycle causing LNCaP cells to shift into the sub-G0 apoptotic fraction and the PC-3 cells to arrest in the G2/M phase. Second, the LNCaP cell line is approximately two fold more sensitive to F-4 induction of apoptosis and cytotoxicty compared to PC-3 cells. These observations, combined with the common and unique effects of F-4 on client proteins, suggest novobiocin analogues may exert cell type specific modes of action. Lastly, 17-AAG treatment of prostate cancer cells at concentrations of one and 50 micromolar appeared to be largely cytostatic causing a G2/M arrest with mild to modest induction of apoptosis and cell death.

In conclusion, the novobiocin analogue, F-4, is a putative C-terminal Hsp90 inhibitor with distinct pharmacology compared to the N-terminal inhibitor 17-AAG. At IC_{90} anti-proliferative concentrations, F-4 is superior to 17-AAG at inducing apoptosis and cytotixicty and may offer advantages over N-terminal inhibitors in the treatment of prostate cancer.

Acknowledgments

The authors gratefully acknowledge the support of this project by Department of Defense New Investigator Award, PC050629 of the Prostate Cancer Research Program and NIH (CA120458). This work was supported in part by the Kansas Technology Enterprise Corporation through the Centers of Excellence Program.

References

1. Jemal, A., Tiwari, R. C., Murray, T. et al.: Cancer statistics, 2004. CA Cancer J Clin, **54:** 8, 2004

2. Petrylak, D. P., Tangen, C. M., Hussain, M. H. et al.: Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. N Engl J Med, **351:** 1513, 2004

3. Tannock, I. F., de Wit, R., Berry, W. R. et al.: Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med, **351:** 1502, 2004

4. Stravopodis, D. J., Margaritis, L. H., Voutsinas, G. E.: Drug-mediated targeted disruption of multiple protein activities through functional inhibition of the Hsp90 chaperone complex. Curr Med Chem, **14:** 3122, 2007

5. Neckers, L.: Heat shock protein 90: the cancer chaperone. J Biosci, **32:** 517, 2007

6. Chiosis, G., Huezo, H., Rosen, N. et al.: 17AAG: low target binding affinity and potent cell activity--finding an explanation. Mol Cancer Ther, **2:** 123, 2003

7. Kamal, A., Thao, L., Sensintaffar, J. et al.: A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature, **425:** 407, 2003

8. Marcu, M. G., Chadli, A., Bouhouche, I. et al.: The heat shock protein 90 antagonist novobiocin interacts with a previously unrecognized ATP-binding domain in the carboxyl terminus of the chaperone. J Biol Chem, **275:** 37181, 2000

9. Whitesell, L., Shifrin, S. D., Schwab, G. et al.: Benzoquinonoid ansamycins possess selective tumoricidal activity unrelated to src kinase inhibition. Cancer Res, **52:** 1721, 1992

10. Burlison, J. A., Blagg, B. S.: Synthesis and evaluation of coumermycin A1 analogues that inhibit the Hsp90 protein folding machinery. Org Lett, **8:** 4855, 2006

11. Galam, L., Hadden, M. K., Ma, Z. et al.: High-throughput assay for the identification of Hsp90 inhibitors based on Hsp90-dependent refolding of firefly luciferase. Bioorg Med Chem, **15:** 1939, 2007

12. Yu, X. M., Shen, G., Neckers, L. et al.: Hsp90 inhibitors identified from a library of novobiocin analogues. J Am Chem Soc, **127:** 12778, 2005

13. Burlison, J. A., Avila, C., Vielhauer, G. et al.: Development of novobiocin analogues that manifest anti-proliferative activity against several cancer cell lines. J Org Chem, **73:** 2130, 2008

14. Goldson TM, V. G., Staub E, Miller S, Shim H, Hagedorn CH: Eukaryotic initiation factor 4E variants alter the morphology, proliferation, and colony-formation properties of MDA-MB-435 cancer cells. Molecular Carcinogenesis, **46:** 71, 2007

15. Son D, R. K. a. T. P.: Tumor necrosis factor alpha (TNF) induces serum amyloid A3 in mouse granulosa cells Endocrinology, **145:** 2245, 2004

16. Zhang, H., Burrows, F.: Targeting multiple signal transduction pathways through inhibition of Hsp90. J Mol Med, **82:** 488, 2004

17. Agoulnik, I. U., Weigel, N. L.: Androgen receptor action in hormone-dependent and recurrent prostate cancer. J Cell Biochem, **99:** 362, 2006

18. Senju, M., Naoko, S., Sato, A. et al.: Hsp90 inhibitors cause G2/M arrest associated with the reduction of Cdc25C and Cdc2 in lung cancer cell lines. J Cancer Res Clin Oncol, **132:** 150, 2006

Figure Legends:

Figure 1

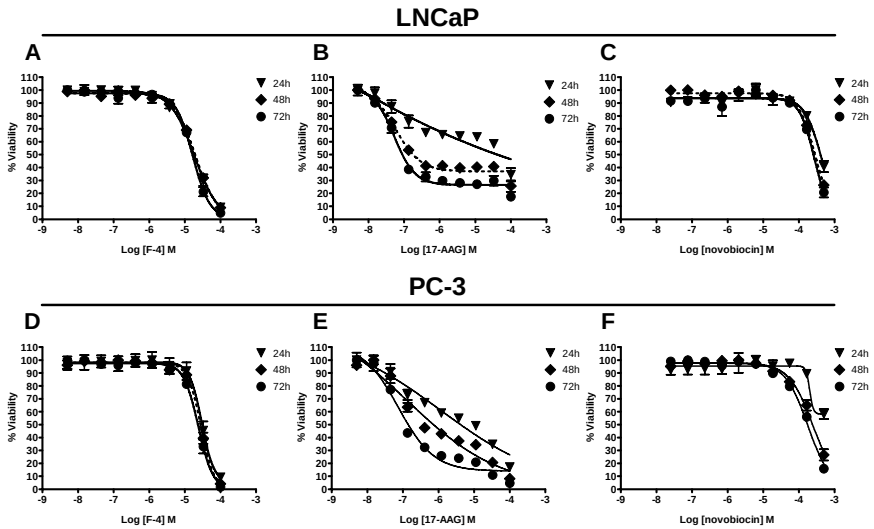


Figure One: *Anti-proliferative effects following dose-response treatment of prostate cancer cells with F-4, 17-AAG, and novobiocin.* Average IC_{50} and IC_{90} values *(Table 1)* were calculated from dose response curves following 24(▼), 48(◆) and 72(●) hour treatments of LNCaP *(top panels A-C)* and PC-3 cells *(bottom panels D-F)*. Combined data from independent experiments is shown for cells treated with F-4 (*panels A and D*), 17-AAG (*panels B and E*), and novobiocin *(panels C and F).*

Figure 2

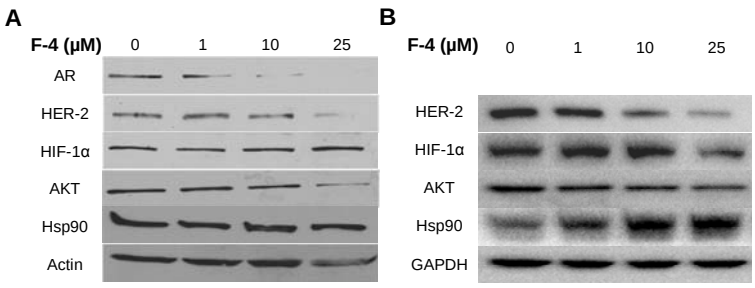


Figure Two: *Degradation of Hsp90 client proteins in prostate cancer cells treated with F-4.* LNCaP (*panel A*) and PC-3 (*panel B*) cells were treated with F-4 then examined by Western blot for the degradation of client proteins. Actin (LNCaP) or GAPDH (PC-3) were used as loading controls for each panel.

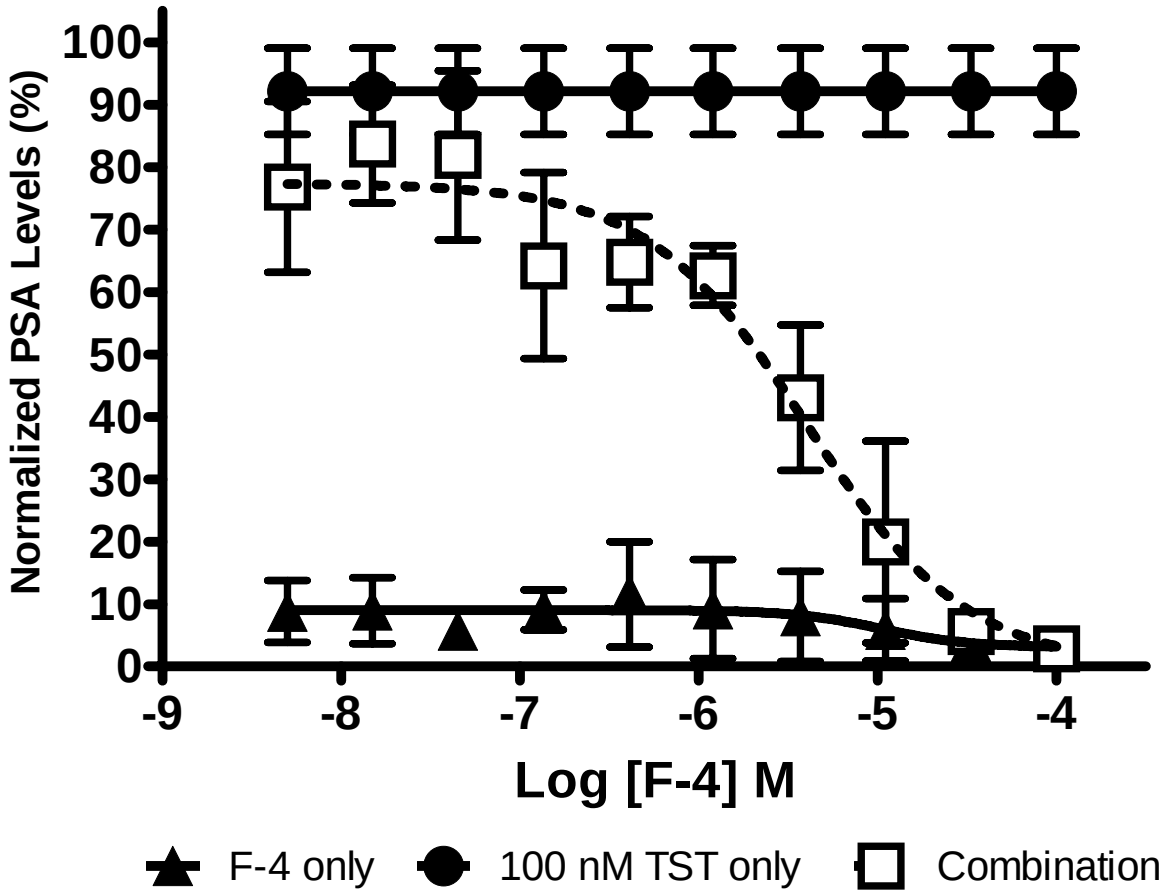


Figure Three: *F-4-mediated reduction of PSA secretion in LNCaP cells.* LNCaP cells were grown in hormone-free media for 72 hours, pre-treated with F-4 for 24 hours then were incubated either with F-4(▲), 100 nM testosterone (TST) (●), or in combination with F-4 (□)**.**

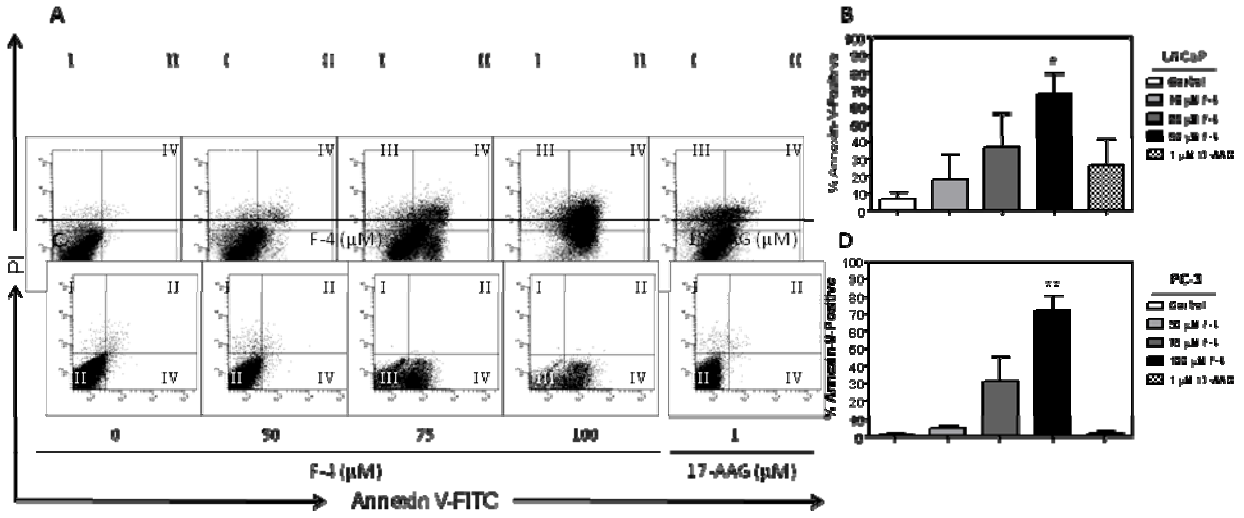


Figure Four: *F-4 induction of apoptosis in prostate cancer cells.* LNCaP and PC-3 cells were treated with F-4 or 17-AAG for 72h. Representative flow cytometry scatterplots depict percent Annexin V staining for LNCaP *(panel A)* and PC-3 cells *(panel C)* after F-4 and 17-AAG treatment. The four quadrants of the scatterplot represent the percent of the parent population and indicate the following: *I.* PI- staining only indicating necrosis; *II.* Annexin V and PI staining depicting late-stage apoptosis; *III.* Negative staining indicating live cells; *IV.* Annexin V staining alone indicating early-stage apoptosis. A bar graph depicting the total percent apoptosis (Annexin V positive cells in quadrants II + IV) following treatment with statistical analysis is shown for LNCaP and PC-3 cells (*panels B and D, respectively*). Asterisk(s) *, ** indicates significant *P value <0.05, and <0.01, respectively* by t-test compared to vehicle-treated control.

Figure 5

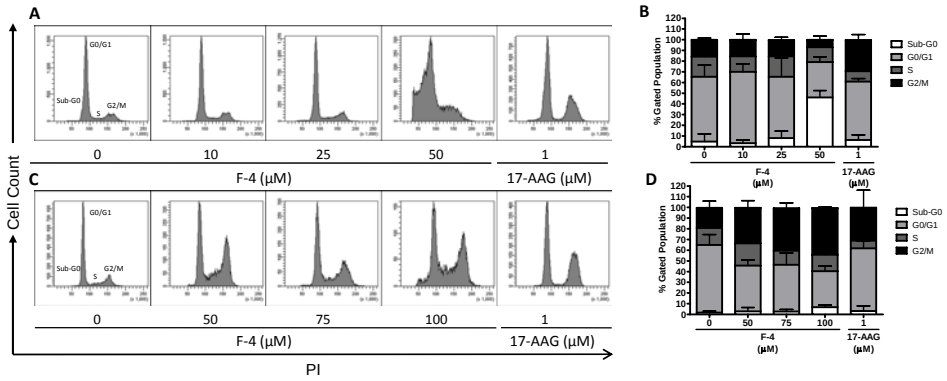


Figure Five: *Cell cycle effects of F-4 in prostate cancer cells*. Representative histograms are shown depicting the distribution of cell cycle following treatment of LNCaP (*panel A*) and PC-3 (*panel C*) cells with F-4 or 17-AAG for 72h. Bar graphs depicting the cell cycle distribution after treatment from three independent experiments is shown with statistical analysis for LNCaP and PC-3 (*panels B and D, respectively).*

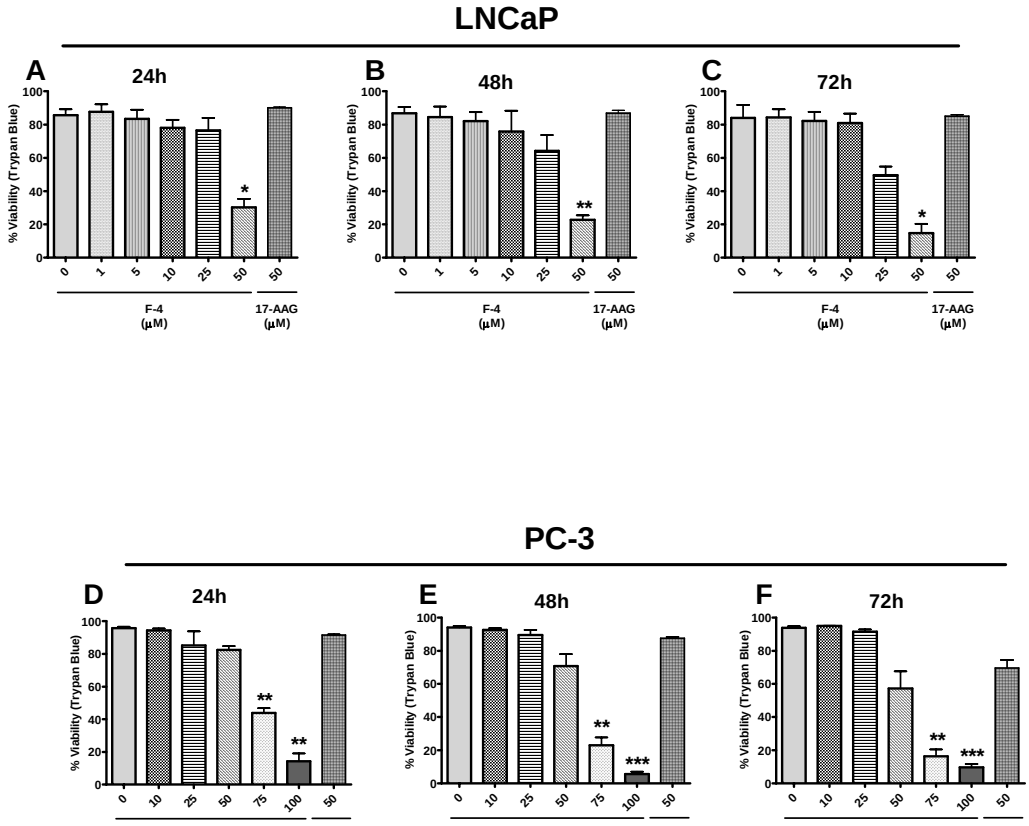


Figure Six: *Cytotoxic effects of F-4 in prostate cancer cell lines.* LNCaP and PC-3 cells were treated with F-4 for 24, 48, and 72 hours. Samples were mixed with equal parts Trypan Blue and assessed for viability. Percentage viability was calculated as described in **Materials and Methods.** Asterisk(s), *, **, *** indicates significant *P value <0.05, <0.01, and <0.001, respectively* by t-test compared to vehicle-treated control.